

# Combined Blockade of ADP Receptors and PI3-Kinase p110 $\beta$ Fully Prevents Platelet and Leukocyte Activation during Hypothermic Extracorporeal Circulation

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## Abstract

Extracorporeal circulation (ECC) and hypothermia are used to maintain stable circulatory parameters and improve the ischemia tolerance of patients in cardiac surgery. However, ECC and hypothermia induce activation mechanisms in platelets and leukocytes, which are mediated by the platelet agonist ADP and the phosphoinositide-3-kinase (PI3K) p110 $\beta$ . Under clinical conditions these processes are associated with life-threatening complications including thromboembolism and inflammation. This study analyzes effects of ADP receptor P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> blockade and PI3K p110 $\beta$  inhibition on platelets and granulocytes during hypothermic ECC. Human blood was treated with the P<sub>2</sub>Y<sub>12</sub> antagonist 2-MeSAMP, the P<sub>2</sub>Y<sub>1</sub> antagonist MRS2179, the PI3K p110 $\beta$  inhibitor TGX-221, combinations thereof, or PBS and propylene glycol (controls). Under static *in vitro* conditions a concentration-dependent effect regarding the inhibition of ADP-induced platelet activation was found using 2-MeSAMP or TGX-221. Further inhibition of ADP-mediated effects was achieved with MRS2179. Next, blood was circulated in an *ex vivo* ECC model at 28°C for 30 minutes and various platelet and granulocyte markers were investigated using flow cytometry, ELISA and platelet count analysis. GPIIb/IIIa activation induced by hypothermic ECC was inhibited using TGX-221 alone or in combination with P<sub>2</sub>Y blockers ( $p < 0.05$ ), while no effect of hypothermic ECC or antiplatelet agents on GPIIb/IIIa and GPIIb $\alpha$  expression and von Willebrand factor binding was observed. Sole P<sub>2</sub>Y and PI3K blockade or a combination thereof inhibited P-selectin expression on platelets and platelet-derived microparticles during hypothermic ECC ( $p < 0.05$ ). P<sub>2</sub>Y blockade alone or combined with TGX-221 prevented ECC-induced platelet-granulocyte aggregate formation ( $p < 0.05$ ). Platelet adhesion to the ECC surface, platelet loss and Mac-1 expression on granulocytes were inhibited by combined P<sub>2</sub>Y and PI3K blockade ( $p < 0.05$ ). Combined blockade of P<sub>2</sub>Y<sub>12</sub>, P<sub>2</sub>Y<sub>1</sub> and PI3K p110 $\beta$  completely inhibits hypothermic ECC-induced activation processes. This novel finding warrants further studies and the development of suitable pharmacological agents to decrease ECC- and hypothermia-associated complications in clinical applications.

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## Introduction

Under physiological conditions, platelets play a fundamental role in hemostasis, prevention of blood loss, and healing of vascular injury. However, dysfunctional platelets can cause serious problems like abnormal thrombus formation and consecutive vessel occlusion as well as severe bleeding complications, which are all feared side effects of extracorporeal circulation (ECC) [1,2].

ECC is employed in many cardiac surgical procedures to ensure gas exchange and to maintain stable circulatory parameters of the patient. In addition, hypothermia ranging between 28°C and 32°C is routinely employed during cardiac operations in addition to ECC to increase the ischemia tolerance of the patient. Shear stress, contact of blood with the artificial surfaces of the ECC circuit as well as hypothermia are all known to be associated with platelet activation, which results in disturbed platelet function and associated complications [1,3,4]. Furthermore, activated platelets

can trigger an inflammatory response through interactions with leukocytes [5]. These platelet-leukocyte interactions are mainly mediated by binding of the platelet surface receptor P-selectin to its counter receptor P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. Subsequently, upregulation and activation of the Mac-1 receptor (CD11b/CD18) on leukocytes is induced as a result of the P-selectin-PSGL-1 interaction [5,6]. Furthermore, it has been shown that CD40 ligand, which is shed from platelets upon activation, also promotes Mac-1 upregulation [7].

Inhibition of platelet activation is a possible approach to inhibit platelet dysfunction and related detrimental effects during ECC. One pharmacological strategy to inhibit platelet activation is blockade of the platelet ADP receptors P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> [8,9].

We have recently shown that ADP plays a major role in ECC- and hypothermia-induced platelet activation [10]. Inhibition of platelet granule release could be achieved during hypothermic ECC via P<sub>2</sub>Y<sub>12</sub> blockade [11]. Nevertheless, despite effective

platelet protection by  $P_2Y_{12}$  blockade, still higher degrees of platelet activation compared to baseline values were observed. Furthermore, platelet adhesion to the ECC surface and therefore platelet loss could not be prevented. Consequently, in addition to ADP other factors obviously activate platelets during ECC. In this regard, shear-induced activation of platelets is another important factor during ECC [4,12]. Shear triggers a signaling pathway, which includes activation of the class Ia phosphoinositide-3-kinase (PI3Ks) p110 $\beta$  isoform. This results in activation of the platelet fibrinogen receptor GPIIb/IIIa and platelet aggregate formation [13,14,15,16].

On the basis of these data, we hypothesize that substantial platelet protection during ECC and hypothermia may be achieved by combined inhibition of  $P_2Y_{12}$ ,  $P_2Y_1$  and PI3K p110 $\beta$ . To prove this, we first defined effective doses of the  $P_2Y_{12}$  antagonist 2-MeSAMP and the PI3K p110 $\beta$  blocker TGX-221 to achieve substantial inhibition of platelet activation *in vitro*. Afterwards, the effect of single and combined blockade of ADP receptors and PI3K p110 $\beta$  on platelet activation, platelet adhesion to the ECC surface, their interaction with granulocytes as well as subsequent granulocyte activation was investigated in human whole blood employing an *ex vivo* ECC model at hypothermia (28°C).

## Results

### Concentration-dependent Inhibition of ADP-induced P-selectin Expression using 2-MeSAMP and TGX-221 and the Effect of MRS2179

Treatment of whole blood with different concentrations of 2-MeSAMP (10 and 100  $\mu$ M) showed that ADP-induced (final ADP concentration: 20  $\mu$ M) P-selectin expression is more potently inhibited with higher antagonist concentrations (Figure 1A). The addition of MRS2179 (100  $\mu$ M) in the 2-MeSAMP-treated group further decreased the expression of platelet P-selectin expression upon ADP activation (Figure 1A).

Furthermore, a TGX-221 concentration of 2.2  $\mu$ M is more potently inhibiting ADP-induced P-selectin expression in human

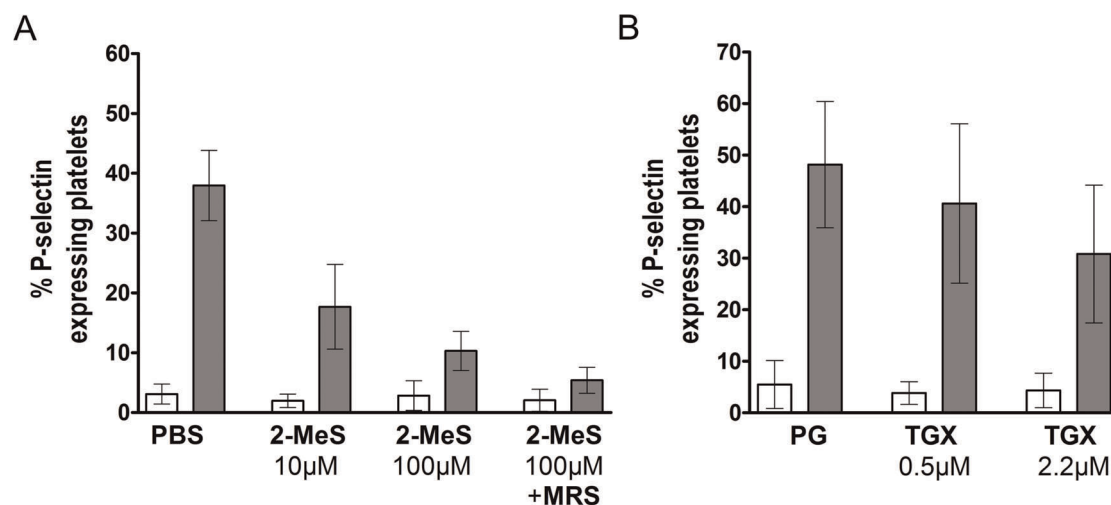
whole blood, when compared to a TGX-221 concentration of 0.5  $\mu$ M (Figure 1B).

### PI3K p110 $\beta$ Inhibition Alone or in Combination with ADP Receptor Blockade has no Effect on Expression Levels of GPIIb/IIIa and GPIb $\alpha$ as Well as on vWF Binding of Platelets, but Reduces GPIIb/IIIa Activation during Hypothermic ECC

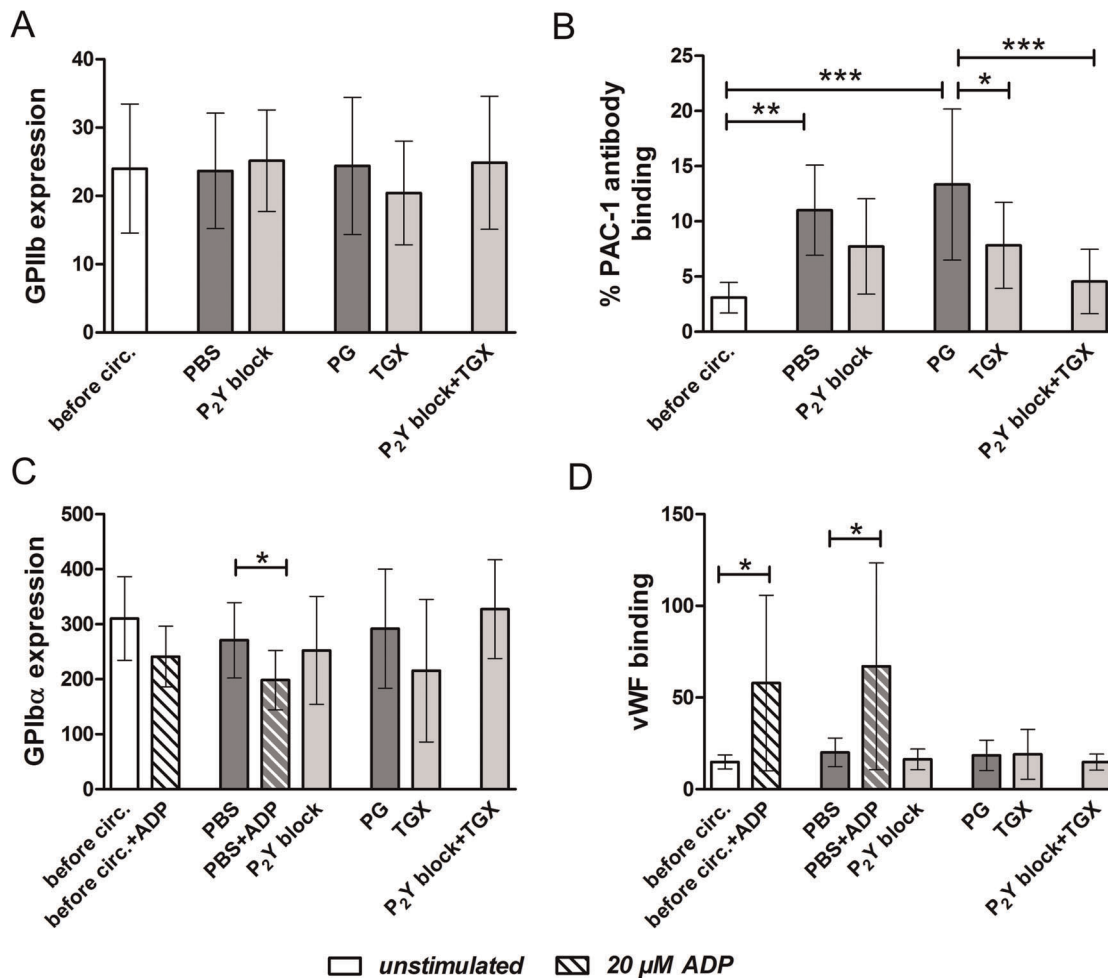
Platelet activation is accompanied by a conformational change of the platelet GPIIb/IIIa receptor into a high-affinity conformation, which enables binding of ligands including fibrinogen and vWF [19]. Using specific antibodies we analyzed (1) the general expression of GPIIb/IIIa on the platelet surface and (2) the GPIIb/IIIa activation state. Basic GPIIb/IIIa expression was neither influenced by hypothermic ECC nor by antiplatelet agents (Figure 2A), while hypothermic ECC induced a significant increase in GPIIb/IIIa activation in controls ( $p < 0.01$ ; Figure 2B). This effect was inhibited by PI3K p110 $\beta$  inhibition using TGX-221 alone ( $p < 0.05$ ) and more profoundly by PI3K p110 $\beta$  inhibition combined with  $P_2Y$  blockade (2-MeSAMP and MRS2179;  $p < 0.001$ ).

The GPIb $\alpha$  receptor is involved in mediating primary platelet adhesion via binding to its ligand vWF [19]. Upon platelet activation GPIb $\alpha$  surface expression is decreased due to down-regulation [20] or receptor shedding [21,22]. Regarding the expression of the GPIb $\alpha$  receptor on platelets as well as vWF binding to platelets in our experiments, no effect of either hypothermic ECC or  $P_2Y$  and PI3K p110 $\beta$  inhibition was observed (Figure 2C and D). It has previously been described that the platelet agonist ADP decreases GPIb $\alpha$  surface expression on platelets [20,23,24]. This finding was confirmed in our experiments and indicated intact platelet reactivity before and after hypothermic ECC in the PBS group (Figure 2C).

In addition, vWF binding was investigated on ADP-activated platelets before and after hypothermic ECC in the PBS group. ADP induced a significant increase in vWF binding at both timepoints (Figure 2D). Regarding the fact that GPIb $\alpha$  surface expression was downregulated at the same time, this finding is



**Figure 1. Concentration-dependent inhibition of ADP-induced P-selectin expression using 2-MeSAMP and TGX-221.** Heparinized human whole blood ( $n = 4$ ) was treated for 5 minutes at 37°C with (A) PBS as control, 2-MeSAMP (10 and 100  $\mu$ M; “2-MeS”) or a combination (100  $\mu$ M each) of 2-MeSAMP and MRS2179 and (B) propylene glycol (“PG”) as control or TGX-221 (0.5 or 2.2  $\mu$ M; “TGX”). Afterwards platelets were activated using ADP (final concentration: 20  $\mu$ M) and the percentage of P-selectin expressing platelets under a pre-set histogram marker was analyzed in flow cytometry using an anti-P-selectin mAb. Data are given as means and SD. doi:10.1371/journal.pone.0038455.g001



**Figure 2. GPIIb/IIIa and GPIb $\alpha$  expression as well as vWF binding are not influenced by *ex vivo* hypothermic ECC and antiplatelet agents, while platelet PI3K p110 $\beta$  inhibition alone or in combination with P<sub>2</sub>Y<sub>12</sub>/P<sub>2</sub>Y<sub>1</sub> receptor blockade reduce GPIIb/IIIa activation induced by hypothermic ECC.** Human blood was left untreated ("before circ.") or treated *ex vivo* with PBS (control), a combination of 2-MeSAMP and MRS2179 to block P<sub>2</sub>Y receptors (100  $\mu$ M each; "P<sub>2</sub>Y block"), propylene glycol ("PG", control), TGX-221 to inhibit PI3K p110 $\beta$  (2.2  $\mu$ M; "TGX") or a combination of 2-MeSAMP, MRS2179 (100  $\mu$ M each) and TGX-221 (2.2  $\mu$ M; "P<sub>2</sub>Y block + TGX"). All treated samples were circulated in an ECC model for 30 minutes at 28°C. Expression of GPIIb (A; n=4), activated GPIIb/IIIa (B; n=6), GPIb $\alpha$  (C; n=6) as well as vWF binding (D; n=4) were evaluated in flow cytometry using specific antibodies. ADP stimulation (20  $\mu$ M) before and after hypothermic ECC was performed as positive control for GPIb $\alpha$  expression (C) and vWF binding (D) in the PBS group. Geometric mean fluorescence values of fluorescently labeled antibodies are given in diagrams as means and SD; not normally distributed data were analyzed using a non-parametrical test (Friedman test with Dunns multiple comparison test; A, D); normally distributed data (B, C) were compared using RM-ANOVA with Bonferroni's multiple comparison test; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. doi:10.1371/journal.pone.0038455.g002

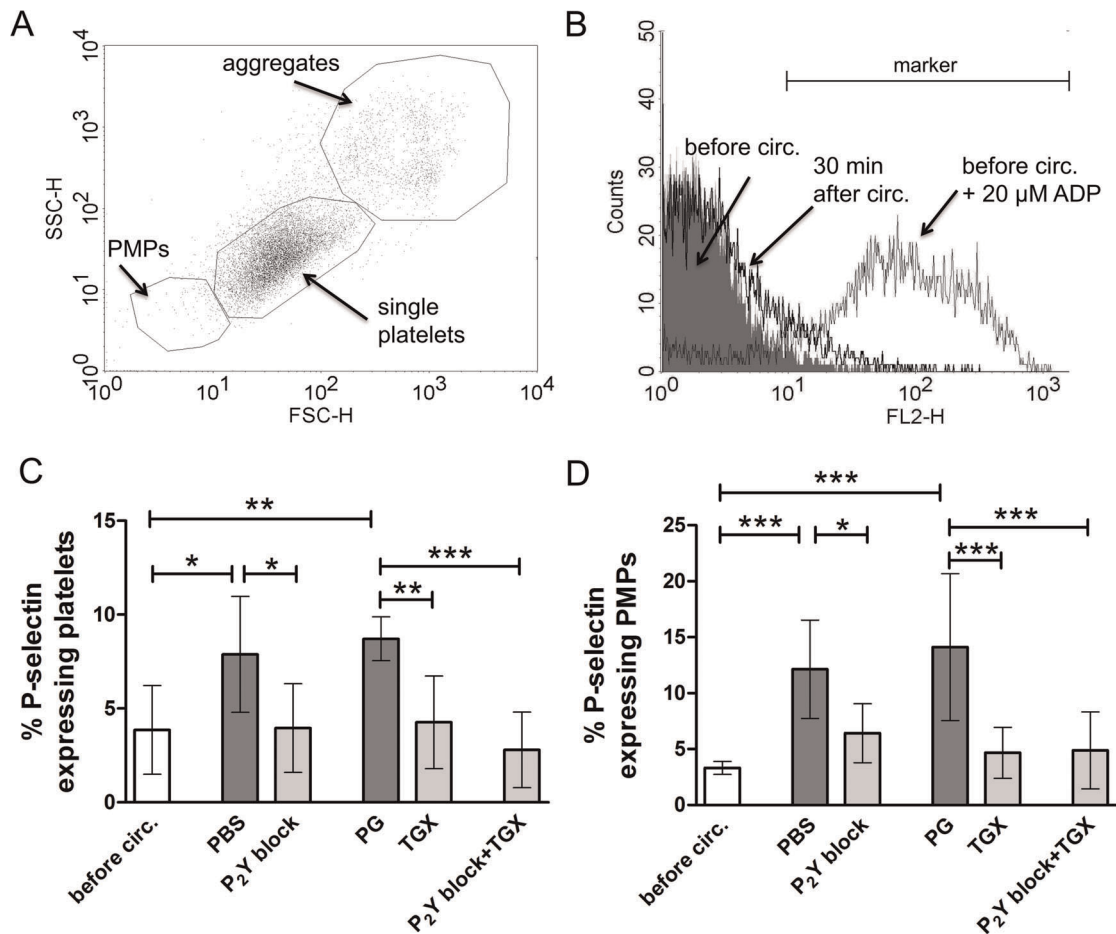
most likely due to binding of vWF to GPIIb/IIIa. In this regard, it has previously been described that platelets have other binding sites for vWF than GPIIb $\alpha$  [25] and that vWF is a ligand of the GPIIb/IIIa receptor [19,26].

#### Hypothermic ECC-induced P-selectin Expression on Platelets and Platelet Microparticles is Profoundly Inhibited by P<sub>2</sub>Y and PI3K p110 $\beta$ Inhibitors

As an indicator for  $\alpha$ -granule release and platelet activation, P-selectin expression on the surface of platelets and platelet-derived microparticles was measured before and after hypothermic ECC. Single platelets and PMPs were identified according to their size and granularity (Figure 3A). As a positive control P-selectin expression on ADP-stimulated platelets was measured to confirm platelet reactivity before and after circulation in the control group (Figure 3B).

P-selectin expression significantly increased during hypothermic ECC in controls (p<0.05, Figure 3C). Both P<sub>2</sub>Y blockade and PI3K p110 $\beta$  inhibition alone as well as combined inhibition of ADP receptors and PI3K p110 $\beta$  completely inhibited this phenomenon (p<0.05).

PMPs, which express typical platelet surface receptors including P-selectin, are released from platelets upon activation [3,27,28]. In our experiments the total number of PMPs was not affected by hypothermic ECC or antiplatelet agents (data not shown). Nevertheless, P-selectin expression on PMPs was significantly increased by hypothermic ECC (p<0.001; Figure 3D). P<sub>2</sub>Y receptor blockade, PI3K p110 $\beta$  inhibition as well as the combination of P<sub>2</sub>Y receptor and PI3K p110 $\beta$  inhibition significantly reduced P-selectin expression on PMPs (p<0.05).



**Figure 3. Blockade of P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> as well as PI3K p110 $\beta$  inhibition profoundly inhibits hypothermic ECC-induced P-selectin expression on platelets and platelet microparticles.** Prior to ("before circ.") and after hypothermic ECC (28°C, 30 minutes) flow cytometric analysis of P-selectin expression on platelets and PMPs was performed in groups treated with either PBS as control, 2-MeSAMP and MRS2179 (100  $\mu$ M each; "P<sub>2</sub>Y block"), propylene glycol ("PG") as control, TGX-221 to inhibit PI3K p110 $\beta$  (2.2  $\mu$ M; "TGX") or a combination of 2-MeSAMP and MRS2179 (100  $\mu$ M each) as well as TGX-221 (2.2  $\mu$ M; "P<sub>2</sub>Y block + TGX"). Representative dot plot indicating the identification of PMPs, single platelets and aggregates according to their size and granularity (A). Representative histogram overlay including a marker to identify percentages of P-selectin expressing platelets before circulation without additional stimulation (grey filled), before circulation with addition of ADP (20  $\mu$ M; grey solid line) as well as 30 minutes after hypothermic ECC (black solid line) (B). Percentages of platelets (C) and PMPs (D) expressing P-selectin are depicted. Data in (C) and (D) are given as means (n=6) and SD; groups were compared using RM-ANOVA with Bonferroni's multiple comparison test; \*p<0.05; \*\*p<0.01, \*\*\*p<0.001.

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### Effects of Hypothermic ECC, P<sub>2</sub>Y Blockade and PI3K p110 $\beta$ Inhibition on Platelet-ECC Adhesion, Platelet-granulocyte Aggregate Formation and Platelet Loss

Hypothermic ECC induced a significant increase in platelet adhesion to the artificial ECC surface in controls (p<0.001; Figure 4A). This was only prevented by a combination of P<sub>2</sub>Y blockade and PI3K p110 $\beta$  inhibition (p<0.05), but not by P<sub>2</sub>Y blockade or PI3K p110 $\beta$  inhibition alone.

Interaction of activated platelets with leukocytes plays a pivotal role in ECC-related pro-inflammatory complications [1,29]. We evaluated the formation of platelet-granulocyte aggregates during hypothermic ECC *ex vivo*. A significant increase in aggregate formation was found after 30 minutes of circulation at 28°C in controls (p<0.01; Figure 4B). This phenomenon was significantly inhibited by P<sub>2</sub>Y blockade alone (p<0.05) and by combined treatment with P<sub>2</sub>Y and PI3K p110 $\beta$  blockers (p<0.05).

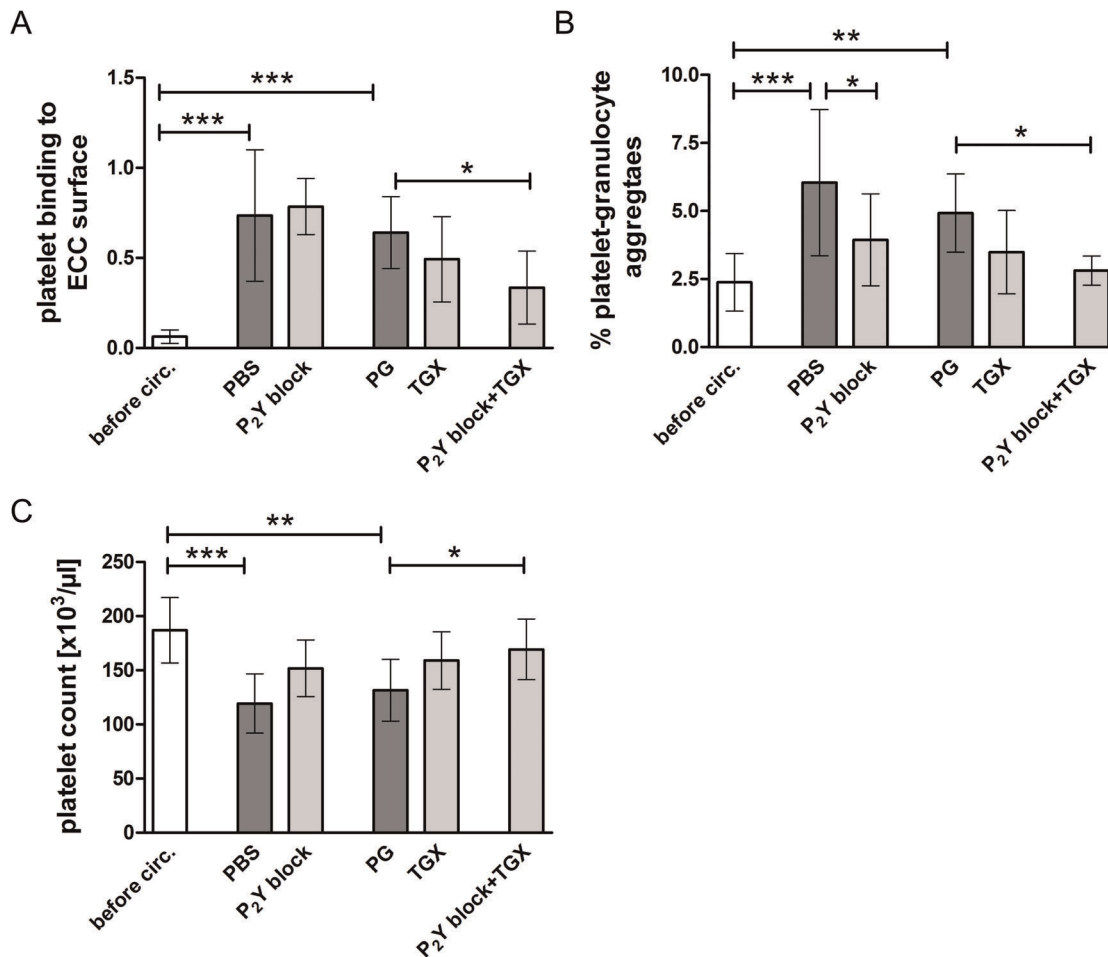
A significant decrease of platelet counts was observed during hypothermic ECC (p<0.01; Figure 4C). The combination of P<sub>2</sub>Y

blockers and PI3K p110 $\beta$  inhibitor significantly prevented hypothermic ECC-induced platelet loss (p<0.05).

### P<sub>2</sub>Y Blockade in Combination with a PI3K p110 $\beta$ Inhibitor Prevents Upregulation of the Mac-1 Receptor on Granulocytes

Interaction of activated platelets with leukocytes triggers the upregulation of the Mac-1 receptor on leukocytes. Mac-1 mediates leukocyte adhesion and migration as well as interaction and binding of leukocytes and platelets [30]. To evaluate granulocyte activation, we measured surface CD11b expression on granulocytes. Hypothermic ECC induced a profound increase in CD11b expression on granulocytes in controls (p<0.05; Figure 5). This was significantly decreased by P<sub>2</sub>Y blockade in combination with PI3K p110 $\beta$  inhibition (p<0.001). P<sub>2</sub>Y and PI3K p110 $\beta$  inhibition alone had no effect on hypothermic ECC-induced CD11b expression on granulocytes.





**Figure 4. Effects of hypothermic ECC and platelet inhibitor treatment on platelet adhesion to the ECC surface, platelet-granulocyte aggregate formation and platelet counts.** Human blood samples were either left untreated or treated with PBS (control group), a combination of 2-MeSAMP and MRS2179 (100  $\mu\text{M}$  each; "P<sub>2</sub>Y block"), propylene glycol ("PG", control group), TGX-221 (2.2  $\mu\text{M}$ ; "TGX") or a combination of 2-MeSAMP, MRS2179 and TGX-221 ("P<sub>2</sub>Y block + TGX"). Blood, which was treated accordingly, was circulated in an *ex vivo* ECC model at 28°C for 30 minutes. A specific ELISA method was employed to detect platelet adhesion to the ECC surface (A;  $n=6$ ). Platelet-granulocyte aggregate formation was measured in flow cytometry before circulation ("before circ.") and after circulation in all treatment groups (B;  $n=4$ ) according to the fluorescence of an anti-CD15-PE antibody on aggregates (aggregate region in figure 3A) under a pre-set histogram marker. Platelet counts were measured in all samples (C;  $n=6$ ). Data are given as means and SD; normally distributed data were compared using RM-ANOVA with Bonferroni's multiple comparison test (A, B); not normally distributed data were analyzed using a non-parametrical test (Friedman test with Dunns multiple comparison test; C); \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ . doi:10.1371/journal.pone.0038455.g004

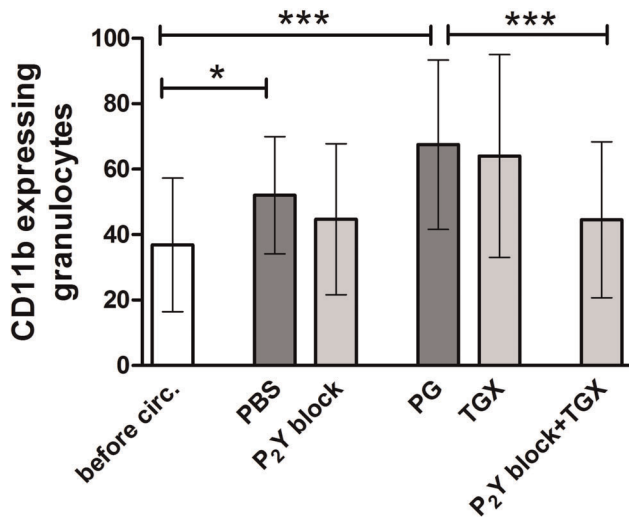
## Discussion

In this study, we investigate the effects of ADP receptor blockade combined with PI3K p110 $\beta$  inhibition on platelets and granulocytes in an *ex vivo* model simulating hypothermic ECC. In general, our current findings confirm previously reported effects of hypothermic ECC on platelet activation, which lead to numerous responses in platelets like: platelet adhesion to the ECC surface, granule release associated with upregulation of P-selectin on platelets and PMPs, activation of GPIIb/IIIa and subsequent interaction of platelets and granulocytes as well as granulocyte activation (Figure 6) [1,2,12,31,32,33,34]. In our current study, GPIIb $\alpha$  and GPIIb/IIIa expression levels as well as vWF binding to platelets were unaffected by hypothermic ECC. Hypothermic ECC did also not result in increased PMP numbers, but in an increase of P-selectin expression on PMPs and single platelets. This contributes to explain the increase in platelet-granulocyte aggregate formation and upregulation of Mac-1 expression on

granulocytes as observed in our experiments, since P-selectin expressing platelets and PMPs can mediate binding and consecutive activation of platelets and leukocytes [35]. Furthermore, the observed loss of platelet counts after hypothermic ECC can be explained by the fact that aggregate formation as well as platelet adhesion to the ECC surface occurred during hypothermic ECC.

Based on these data and previous findings [3,10,36], a routine strategy for platelet protection during ECC and hypothermia is warranted and could be very beneficial for many patients in clinical settings where ECC and hypothermia are employed.

In patients with coronary disease platelet inhibition is routinely performed with ADP receptor P<sub>2</sub>Y<sub>12</sub> blockers including the thienopyridines clopidogrel and prasugrel [37,38]. We have recently reported that administration of cangrelor, a reversible non-thienopyridine P<sub>2</sub>Y<sub>12</sub> receptor blocker with short plasma half-life (3–6 minutes) [39] effectively reduces platelet granule release, platelet-granulocyte binding, and platelet loss during hypothermic ECC [11]. Furthermore, according to its short half-



**Figure 5. P<sub>2</sub>Y<sub>12</sub>/P<sub>2</sub>Y<sub>1</sub> blockade in combination with TGX-221 prevents upregulation of the Mac-1 receptor on granulocytes.** Granulocyte activation was measured before ("before circ.") and 30 minutes after hypothermic ECC in groups treated with PBS (control group), 2-MeSAMP and MRS2179 ("P<sub>2</sub>Y block"; 100  $\mu$ M each), propylene glycol ("PG", control group), TGX-221 ("TGX"; 2.2  $\mu$ M) and a combination of P<sub>2</sub>Y block (100  $\mu$ M each) and PI3K p110 $\beta$  inhibition with TGX-221 (2.2  $\mu$ M; "P<sub>2</sub>Y block + TGX"). Mac-1 expression on granulocytes was evaluated using geometric mean values of antibody fluorescence in flow cytometry. Data are given as means (n=6) and SD; groups were compared using RM-ANOVA with Bonferroni's multiple comparison test; \*p<0.05; \*\*\*p<0.001.

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life administration of cangrelor only during ECC results in rapid recovery of platelet function after ECC and is not associated with blood loss. Nevertheless, cangrelor had no inhibitory effects on platelet-ECC binding and no sustainable effect on GPIIb/IIIa activation [11]. In the setting of normothermic *ex vivo* ECC (37°C), platelet-ECC adhesion as well as platelet-platelet and platelet-granulocyte aggregation can be very effectively decreased with the PI3K p110 $\beta$  inhibitor TGX-221 [4]. In addition, further inhibition of ADP-mediated effects may be achieved by blocking not only P<sub>2</sub>Y<sub>12</sub>, but also P<sub>2</sub>Y<sub>1</sub> on platelets. Therefore, the aim of our current study was to combine full platelet ADP receptor blockade with inhibition of shear-induced platelet activation to completely inhibit platelet activation during ECC and hypothermia.

At first, we employed a combination of experimental P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> blockers to protect platelets during hypothermic ECC. The P<sub>2</sub>Y<sub>1</sub> receptor has been suggested as a crucial target for inhibition of ADP-mediated effects, since P<sub>2</sub>Y<sub>1</sub> activation is necessary for full platelet aggregation [8]. Nevertheless, until today, no P<sub>2</sub>Y<sub>1</sub> antagonist for clinical applications has been developed [40,41]. We therefore used the experimental and reversible P<sub>2</sub>Y<sub>1</sub> antagonist MRS2179, which has been shown to substantially inhibit ADP-induced platelet aggregation [42]. Furthermore, 2-MeSAMP, a specific inhibitor of the platelet P<sub>2</sub>Y<sub>12</sub> receptor [43,44], was used in the current study, because previous *ex vivo* experiments as well as our current *in vitro* experiments showed substantial and specific P<sub>2</sub>Y<sub>12</sub> blockade to a similar extent then the effect observed with cangrelor [10,11].

Our results show, that combined P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> blockade results in significant inhibition of P-selectin expression on platelets and PMPs as well as a reduction in platelet-granulocyte aggregates. Nevertheless, similar to results observed in a previous

study [11], no effect of P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> blockade was observed for GPIIb/IIIa activation and platelet-ECC adhesion.

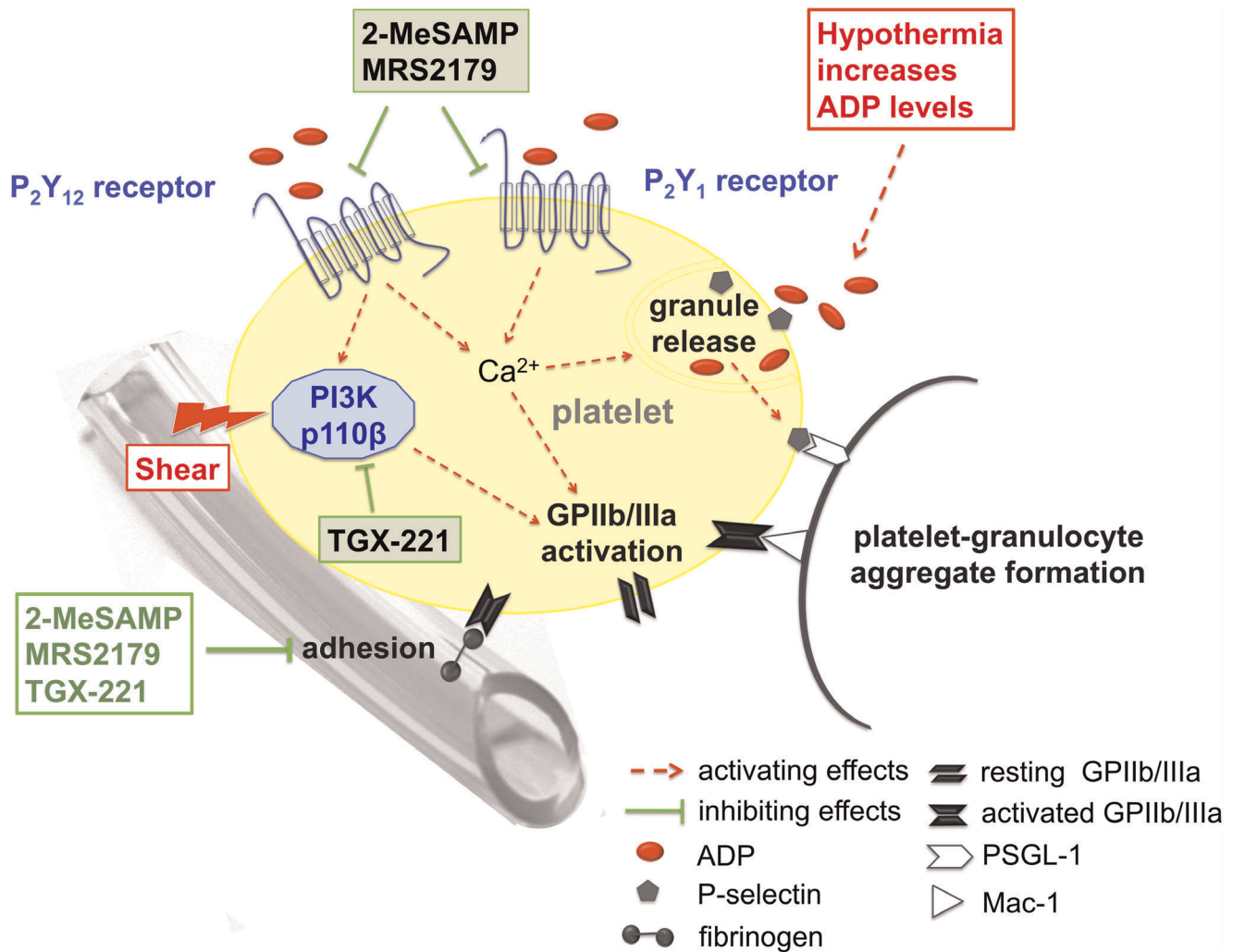
Jackson *et al.* have reported the platelet PI3K p110 $\beta$  isoform as important target for new antithrombotic agents, since it plays a major role in shear-induced activation of platelets [45]. In order to inhibit the platelet PI3K isoform p110 $\beta$ , TGX-221, an experimental compound with a reported 1.000-fold selectivity over the two other PI3K isoforms p110 $\alpha$  and p110 $\gamma$  [45], was used. Only the combined administration of P<sub>2</sub>Y blockade and PI3K p110 $\beta$  inhibition prevented platelet-ECC adhesion during hypothermic ECC and provided a better protection of platelet counts and inhibition of GPIIb/IIIa activation then P<sub>2</sub>Y blockers or TGX-221 alone.

Overall, combined blockade of P<sub>2</sub>Y<sub>12</sub>, P<sub>2</sub>Y<sub>1</sub> and PI3K p110 $\beta$  during hypothermic ECC in our current experiments resulted in an effective reduction of all investigated platelet activation markers. Furthermore, the effect of combined blockade of P<sub>2</sub>Y<sub>12</sub>, P<sub>2</sub>Y<sub>1</sub> and PI3K p110 $\beta$  achieved inhibitory effects close to values measured at baseline before ECC and is therefore more profound than the sole inhibition of P<sub>2</sub>Y<sub>12</sub>, P<sub>2</sub>Y<sub>1</sub> or PI3K p110 $\beta$ . This confirms that activation processes mediated via ADP and shear stress in combination are the major players for the induction of platelet activation during hypothermic ECC. Therefore, inhibition of the respective receptors and activation pathways may be very beneficial for patients undergoing hypothermic ECC. As a consequence the development of respective agents for use in clinical applications is highly desirable. Such innovative substances for administration during ECC and hypothermia should optimally have a short half-life. In this case platelet inhibition can be achieved only during the phase of ECC and hypothermia and platelet function is quickly restored after termination of infusion after ECC. We have recently demonstrated the functionality of this innovative pharmacological principle by employing cangrelor during hypothermic ECC *in vivo* [11].

In conclusion, our novel findings indicate that hypothermic ECC-induced platelet activation as well as subsequent events including aggregate formation and granulocyte activation are completely inhibited by combined P<sub>2</sub>Y<sub>12</sub>, P<sub>2</sub>Y<sub>1</sub> and PI3K p110 $\beta$  blockade. This therapeutic strategy carries the potential to effectively protect platelets and to prevent life-threatening complications like thrombosis, bleeding and systemic inflammation in the setting of ECC and hypothermia. Hence, our findings warrant additional studies to further evaluate this new antiplatelet strategy and to develop innovative reversible antiplatelet agents for clinical use.

### Limitations and Outlook

The Chandler loop ECC model, which was used for the *ex vivo* ECC experiments in this study, is a well-established and routinely used model to analyze effects of ECC and hypothermia on platelets and other blood components. However, the obtained results need to be interpreted with caution, since the Chandler loop model differs in some points from the setting of ECC as employed in cardiac surgery. In the Chandler loop PVC tubings, which contain human whole blood, are circulated in a water bath at a designated temperature. Therefore, human whole blood is circulated exclusively and continuously over artificial surfaces, which causes activation of platelets and leukocytes. However, in the Chandler loop model heart-lung machine components like oxygenator and roller pumps, which are routinely used during cardiac surgery, are omitted and therefore shear stress and other activating effects might be decreased in comparison to what happens under clinical conditions. Furthermore, no priming volume is used in the Chandler loop model, which may result in



**Figure 6. Overview of a pharmacological strategy for platelet protection during hypothermic ECC employing  $P_2Y_{12}$  and  $P_2Y_1$  receptor blockers as well as a PI3K p110 $\beta$  inhibitor.**  
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augmented activating effects. Nevertheless, it has been shown in previous studies that platelet activation is induced during cardiac operations employing a heart-lung machine and hypothermia to a similar extent compared to what we found in our experiments [1,36,46,47].

Our findings indicate that combined  $P_2Y$  and PI3K inhibition has the potential to achieve complete platelet inhibition during hypothermic ECC and may therefore be very beneficial in the clinical situation. Therefore, our final aim is to evaluate this antiplatelet strategy in a carefully designed clinical study. However, previous studies employing different animal models revealed that there is a species-dependent variation regarding the effect of TGX-221 on bleeding [45,48]. Therefore, before a clinical investigation may be initiated, the effect of our experimental strategy on the bleeding time needs to be carefully evaluated in human blood. Especially administration of short-acting drugs like cangrelor or drugs, which don't influence the bleeding time, will be the agents of choice for the *in vivo* setting and should be further evaluated in the future.

## Methods

### Blood Sampling

All blood sampling procedures were approved by the Research and Ethics Unit of the University of Tübingen, Germany (project number 270/2010BO1). Written informed consent was obtained from all subjects before blood sampling.

Blood from healthy donors was collected by venipuncture and anticoagulated with heparin [final concentration (fc): 3 I.U./ml]. All subjects were free of platelet-affecting drugs for at least 14 days.

### *In vitro* Evaluation of Different Concentrations of 2-MeSAMP and TGX-221

Heparinized human whole blood ( $n = 4$ ) was treated for 5 minutes at 37°C with the following agents: PBS as control (Invitrogen GmbH, Karlsruhe, Germany), 2-MeSAMP (fc: 10 and 100  $\mu$ M; dissolved in PBS; Sigma-Aldrich Corporation, St. Louis, USA), a combination of 2-MeSAMP and MRS2179 (fc: 100  $\mu$ M each; dissolved in PBS; Tocris Biosciences, Bristol, UK), propylene glycol (PG; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as control or TGX-221 (fc: 0.5 or 2.2  $\mu$ M; dissolved in PG; Merck Chemicals Ltd., Nottingham, UK). Afterwards whole blood was incubated with

ADP (fc: 20  $\mu$ M) and a phycoerythrin (PE)-labeled anti-P-selectin monoclonal antibody (mAb) (BD Biosciences, Heidelberg, Germany) as well as an fluorescein isothiocyanate (FITC)-labeled anti-GPIIb mAb (Beckman Coulter, Marseille, France) according to previously described methods for 20 minutes at 37°C [3]. Next, all samples were fixed using CellFix® (BD Biosciences) and flow cytometric analysis was performed within 6 hours.

### Ex vivo ECC Chandler Loop Model

In each experiment, baseline values were measured directly after blood sampling in 20 ml of heparinized blood. A well-established closed-loop ECC model (Chandler loop) [17] and PVC tubings (tubing length: 50 cm, inner diameter: 7,6 mm; wall: 2,3 mm) without additional coating (Jostra, Hirrlingen, Germany) were used to simulate ECC at 28°C. For each donor, five tubings were filled with 20 ml of fresh heparinized whole blood and treated with the following agents: the first and second sub-samples were treated with PBS and PG as controls, respectively. In the third sub-sample, both platelet ADP receptors were blocked (“P<sub>2</sub>Y block”) employing the experimental P<sub>2</sub>Y<sub>12</sub> antagonist 2-MeSAMP (fc: 100  $\mu$ M) and the experimental P<sub>2</sub>Y<sub>1</sub> antagonist MRS2179 (fc: 100  $\mu$ M). In the fourth sub-sample, PI3K p110 $\beta$  was inhibited by TGX-221 (fc: 2.2  $\mu$ M). In the last sub-sample, a combination of P<sub>2</sub>Y blockade and PI3K p110 $\beta$  inhibition was employed. Afterwards, each tubing was closed into a circuit and circulated in a water bath (at 30 rpm) at 28°C for 30 minutes.

In this study, blood was anticoagulated with heparin, because heparin is routinely applied during clinical procedures employing ECC as anticoagulant to prevent activation of plasmatic coagulation during ECC. The degree of potential platelet activating effects of heparin on platelets has been analyzed in a previous study and found to be of minor extent in our *ex vivo* ECC model [3].

### Flow Cytometric Analyses of Blood Samples Before and After ECC

Analyses of human platelets and granulocytes were performed directly after blood sampling (“before circulation”) and after *ex vivo* ECC in all treatment groups. Expression of P-selectin, GPIIb $\alpha$  as well as binding of the PAC-1 mAb against activated GPIIb/IIIa on platelets was measured according to previously described methods [3,10].

Expression of the GPIIb/IIIa receptor on platelets as well as platelet von Willebrand factor (vWF) binding was evaluated in 25  $\mu$ l of diluted whole blood (1:50 dilution in modified Tyrodès buffer) using 5  $\mu$ l of an anti-GPIIb-FITC (Beckman Coulter) and 5  $\mu$ l of an anti-vWF-FITC mAb (1:10 dilution; Abcam, Cambridge, UK), respectively. P-selectin and GPIIb $\alpha$  expression as well as vWF binding were also analyzed on ADP-activated (fc: 20  $\mu$ M) platelets before and after hypothermic ECC in the PBS group as positive controls.

Analysis of platelet microparticles (PMPs) was performed after flow cytometric calibration and setup using a forward scatter/sideward scatter dot plot and 1 and 3  $\mu$ m-sized latex beads (Polysciences, Eppelheim, Germany). PMPs are defined as objects with a size of  $\leq$ 1  $\mu$ m. The number of PMPs was counted and P-selectin expression was analyzed as described previously [3]. Platelet-granulocyte aggregates were detected in 25  $\mu$ l of whole blood, which was incubated for 20 minutes with 5  $\mu$ l of an anti-

GPIIb-FITC mAb and 5  $\mu$ l of an anti-CD15-PE mAb. Afterwards, samples were treated with FACS Lysing Solution (BD Biosciences) to lyse erythrocytes, centrifuged at 200 g for 5 minutes and washed with PBS.

CellFix® (BD Biosciences) was used for sample fixation. Flow cytometry was performed within 6 hours using a FACScan® cytometer (BD Biosciences) according to standard procedures [3,18].

For evaluation of granulocyte activation, 90  $\mu$ l of whole blood was incubated with 10  $\mu$ l of an anti-CD11b-PE mAb (Beckman Coulter) for 20 minutes at 37°C, treated with FACS Lysing Solution, centrifuged at 200 g for 5 minutes and afterwards washed with PBS. All samples were fixed using CellFix®. Within 6 hours after antibody staining, granulocytes were identified according to their size and granularity and analyzed using flow cytometry.

For all samples, suitable isotype antibodies were used to adjust fluorescence amplification settings. A total of 10.000 events were acquired in each sample. If not otherwise indicated, geometric mean fluorescence intensities were used for analyses of flow cytometric data.

### Detection of Platelet Adhesion to the ECC Surface

Platelet adhesion to the ECC surface was detected using a specially designed enzyme-linked immunosorbent assay (ELISA) method as previously described [4]. Briefly, ECC tubings were washed and blocked after circulation and surface-bound GPIIb was detected employing a primary anti-GPIIb antibody (Sigma, Deisenhofen, Germany) and an alkaline phosphatase conjugated secondary antibody (Immunotech/Coulter, Marseille, France). The chromogenic reaction was stopped by addition of NaOH. Light absorbance was determined with an ELISA reader MR 5000 (Dynatech, Denkendorf, Germany) at 405 nm.

### Analysis of Platelet Counts

Before and after circulation, human whole blood was anticoagulated with EDTA (EDTA-Monovette®, Sarstedt, Nümbrecht, Germany) for platelet count analysis using an ABX Micros 60 blood analyzer (Axon Lab AG, Baden-Dättwil, Switzerland).

### Statistical Analysis

Data are depicted as means with standard deviation (SD). In order to test data sets for normality, the Kolmogorov-Smirnov test was performed. Normally distributed data were then analyzed using repeated measures (RM) ANOVA with Bonferroni's multiple comparison test to analyze differences between groups, while not normally distributed data were analyzed using a non-parametrical test (Friedman test with Dunn's multiple comparison test). All analyses were performed using the statistical software program GraphPad Prism (version 5, GraphPad Software, La Jolla, USA). Statistical significance was defined as  $p < 0.05$ .

### Author Contributions

Conceived and designed the experiments: KP HW AS. Performed the experiments: SK JK AS. Analyzed the data: SK JK TG KP HW AS. Wrote the paper: SK AS. Approved the final manuscript version: SK JK TG KP HW AS.

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